JAGGED1/NOTCH3 activation promotes aortic hypermuscularization and stenosis in elastin deficiency

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ABSTRACT

Obstructive arterial diseases including supravalvular aortic stenosis (SVAS), atherosclerosis and restenosis share two important features: an abnormal or disrupted elastic lamellae structure and excessive smooth muscle cells (SMCs). However, the relationship between these pathological features is poorly delineated. SVAS is caused by heterozygous loss-of-function, hypomorphic or deletion mutations in the elastin gene ELN, and SVAS patients and elastin mutant mice display increased arterial wall cellularity and luminal obstructions. Pharmacological treatments for SVAS are lacking as underlying pathobiology is inadequately defined. Herein, using human aortic vascular cells, mouse models as well as aortic samples and SMCs derived from induced pluripotent stem cells of ELN-deficient patients, we demonstrated that elastin insufficiency induced epigenetic changes, upregulating the Notch pathway in SMCs. Specifically, reduced elastin increased levels of γ-secretase, activated NOTCH3 intracellular domain and downstream genes. Notch3 deletion or pharmacological inhibition of γ-secretase attenuated aortic hypermuscularization and stenosis in Eln(−/−) mutants. Eln(−/−) mice expressed higher levels of Notch ligand JAGGED1 (JAG1) in aortic SMCs and endothelial cells (ECs). Finally, Jag1 deletion in SMCs, but not ECs, mitigated the hypermuscular and stenotic phenotype in the aorta of Eln(−/−) mice. Our findings reveal that NOTCH3 pathway upregulation induced pathological aortic SMC accumulation during elastin insufficiency and provide potential therapeutic targets for SVAS.
INTRODUCTION

The vasculature is an intricately arranged network of blood vessels with vascular walls that deliver nutrients and remove waste products from target organs. The arterial wall consists of an inner endothelial cell (EC) lining (tunica intima), smooth muscle cells (SMCs) supported by elastic lamellae (tunica media) and the outermost adventitial layer containing fibroblasts and connective tissue (tunica externa). Elastin is the major component of circumferential elastic lamellae that alternate with rings of SMCs to form lamellar units in the media of large elastic vessels such as the aorta. Excessive and aberrant accumulation of SMCs and cells derived from SMCs are a hallmark of diverse obstructive vascular diseases such as supravalvular aortic stenosis (SVAS), atherosclerosis, restenosis, vein graft failure and pulmonary hypertension (1-6). These diseases as well as during physiological closure of the ductus arteriosus are associated with enhanced SMC proliferation and abnormal or disrupted elastic lamellae structure (7-16). Indeed, SVAS, a devastating pediatric condition with obstruction of large and medium-sized arteries, results from loss-of-function, hypomorphic or deletion mutations of one ELN allele (15, 17-19). Similar to SVAS patients, late stage embryonic or early neonatal Eln\(^{+/-}\) mice have increased vascular wall cellularity and arterial lumen obstructions (20). Eln\(^{+/-}\) mice display thinner and a higher number of elastic lamellae with additional SMC layers; however in contrast to Eln\(^{+/+}\) mice and SVAS patients, Eln\(^{+/-}\) mice do not develop aortic stenosis (15, 21). SVAS occurs as an isolated entity (i.e., non-syndromic) or as an integral part of Williams-Beuren syndrome (WBS), a multi-organ system disorder caused by heterozygous deletion of ~27 genes (including ELN) on chromosome 7 (19). Unfortunately, the mechanistic link between elastin defects and hypermuscularization in vascular diseases remains incompletely understood. As a consequence, there is lack of pharmacological agents that prevent excessive proliferation and
accumulation of SMCs, and major surgery remains the only therapy for vessel obstruction in elastin arteriopathy.

The evolutionarily conserved Notch signaling pathway plays key roles in diverse vascular developmental programs, such as aortic wall morphogenesis, EC tip-stalk dynamics during angiogenesis, and in select vascular diseases (22-30); however, no prior investigations have evaluated the role of the Notch pathway in aortic hypermuscularization or stenosis in the context of elastin insufficiency. Notch signaling is initiated by the binding of transmembrane ligands and receptors on neighboring cells. In mammals, the repertoire of Notch ligands consists of Jagged (JAG) 1, 2, Delta-like ligand 1, 3 and 4 and the receptors are NOTCH1-4. Signaling via JAG1 on ECs and SMCs is implicated in differentiation of arterial wall SMC layers (25-28), and NOTCH3 is highly expressed in human arterial SMCs (22). In Notch3 null mice, major elastic arteries of the trunk (e.g., aorta) are indistinguishable from those of wild type mice whereas smaller caliber arteries in mutants have thinner tunica media, impaired SMC differentiation and incomplete vessel maturation (31). In regard to human diseases, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by NOTCH3 mutations (30), and enhanced NOTCH3 expression in SMCs of small arteries in the lung is associated with pulmonary hypertension (29).

In the current study, we report that NOTCH3 activation and signaling are upregulated in cultured human aortic (ha)SMCs with ELN silencing, in the aortic media of both Eln<sup>−/−</sup> mice and WBS patients as well as in WBS and non-syndromic SVAS induced pluripotent stem cell (iPSC)–derived SMCs. Upon engaging ligand, the transmembrane Notch receptor is cleaved by the enzyme γ-secretase, releasing the Notch intracellular domain (NICD) and liberating it to enter the nucleus and modulate gene expression (32). Our results indicate that elastin depletion
results in increased SMC levels of JAG1, the $\gamma$-secretase complex, the activated form of NICD3 and downstream target genes. Mechanistically, SMCs lacking elastin have reduced global DNA methylation - an epigenetic mark that drives gene silencing (33, 34) - and decreased DNA methyltransferase 1 (DNMT1). In particular, elastin depletion in SMCs decreases DNA methylation mark at the promoters of key Notch pathway genes JAG1 and $\gamma$-secretase catalytic subunits PSEN1 and PSEN2, facilitating their upregulation. Moreover, pharmacological inhibition of $\gamma$-secretase or genetic deletion of Notch3 attenuates aortic hypermuscularization and stenosis in Eln$^{-/-}$ mice as well as abrogates excessive muscularization in Eln$^{+/+}$ mice. We previously reported that integrin $\beta3$ plays a key role in elastin aortopathy (3). Herein, our data indicate that NOTCH3 regulates integrin $\beta3$ expression in haSMCs and in mice, and NICD3 binds the promoter of ITGB3, the gene encoding integrin $\beta3$, and these effects are enhanced by elastin deficiency. The initial paper describing that Eln deletion results in arterial stenosis reported a lack of evidence for EC activation or damage (20). Interestingly, our results suggest that EC JAG1 protein is increased in Eln$^{-/-}$ mice at least partly through the effects of the extracellular matrix (ECM) deposited by elastin deficient SMCs. Finally, Jag1 deletion in SMCs, but not in ECs, mitigates the hypermuscularized and stenotic phenotype of the Eln$^{-/-}$ aorta.

Taken together, our data in cultured human cells, mouse models and samples from humans with SVAS and WBS are the first to implicate a role of the Notch signaling pathway and epigenetic remodeling in the pathogenesis of elastin aortopathy and to identify select NOTCH3 pathway members as attractive therapeutic targets for human SVAS and WBS. In addition, these studies provide fundamental mechanistic insights that are integral for advancing potential therapies for a cohort of proliferative and obstructive arterial diseases associated with impaired elastin.
RESULTS

Upregulation of NOTCH3 pathway with loss of elastin

The Notch pathway plays essential roles in SMCs during development and disease of the cardiovascular system (22), but prior studies have not evaluated the role of Notch in aortic hypermuscularization or stenosis in the context of elastin insufficiency. Upon engaging ligand, the transmembrane full-length NOTCH receptor is cleaved by tumor necrosis factor-α-converting enzyme to produce the NOTCH intermediate form (Figure 1A). Subsequent cleavage of the intermediate form by the γ-secretase complex releases NICD into the cytoplasm which translocates into the nucleus, forms a complex with the transcription factor CSL and co-activator Mastermind-like and thereby, modulates gene transcription (22). We initially treated human aortic SMCs (haSMCs) with non-targeting scrambled (Scr) RNA or ELN-specific silencing (si) RNA to query the effect of reduced elastin levels on Notch pathway members. In haSMCs, ELN silencing does not alter transcript levels of the four mammalian NOTCH receptors but does result in a ~4-8-fold increase in levels of key Notch pathway downstream gene products, including hairy and enhancer of split (HES) and Hairy/enhancer-of-split related with YRPW motif protein (HEY) family members (Figure 1B). Among the NOTCH receptors, NOTCH3 is highly expressed in arterial SMCs and not detected in ECs (35-37). To dissect the effect of reduced elastin on NOTCH3 proteolytic processing and activation, lysates collected from ELN-silenced haSMCs were subjected to Western blot analysis (Figure 1C). Elastin knockdown does not change full-length NOTCH3 levels (in agreement with transcript levels in Fig. 1B) but results in a ~2-fold reduction in the NOTCH3 intermediate form with a ~3-4 fold increase in NICD3 and HES1 (Figure 1, C and D). These data suggest that loss of elastin activates the NOTCH3 pathway by inducing the proteolytic cleavage of the NOTCH3 intermediate form.
We next confirmed the upregulation of NICD3 and HES1 with reduced elastin gene dosage in mice and human patient samples. For mice, aortas were isolated from wild type and Eln\(^{-/-}\) pups at postnatal day (P)0.5, and aortic lysates were subjected to Western blot analysis. Eln\(^{-/-}\) aortas have ~3-fold higher protein levels of NICD3 and HES1 as compared to wild type aortas (Figure 1, E and F). NOTCH3 intermediate form did not change in Eln\(^{-/-}\) aortas which might reflect differences in rates of protein synthesis, cleavage and/or degradation in cultured haSMCs versus in vivo (Supplemental Figure 1). Prior studies have shown that NOTCH3 and NOTCH2 having opposing functions in regulating SMC proliferation (38). Interestingly, NOTCH2 protein levels (full length, intermediate form and NICD2) are not altered in Eln\(^{-/-}\) aortas, indicating the specificity of NOTCH3 activation during elastin deficiency (Supplemental Figure 2). Furthermore, to assess the NOTCH3 pathway in human elastinopathy, iPSC-SMC progenitors derived from skin fibroblasts of human control, SVAS or WBS patients were differentiated into SMCs (Supplemental Figure 3). NICD3 and HES1 protein levels are increased in iPSC-SMCs of WBS and non-syndromic SVAS patients compared to those of controls (Figure 1, G and H). Taken together, these data demonstrate that reduced elastin in SMCs results in upregulated NOTCH3 pathway signaling.

**Loss of elastin upregulates \(\gamma\)-secretae complex in SMCs by modulating DNA methylation**

As suggested above, the changes in protein levels of the NOTCH3 intermediate form (reduced) and NICD3 (increased) in haSMCs with elastin silencing implicate \(\gamma\)-secretase-mediated proteolytic cleavage (Figure 1, A, C and D). \(\gamma\)-secretase is a proteosomal complex composed of multiple subunits, including the catalytic presenilins (PSEN1 or 2) and accessory subunits nicastrin (NCT) and presenilin enhancer 2 (PEN2) (39). We next assessed the levels of
γ-secretase complex subunits in elastin deficient haSMCs. siRNA-mediated elastin silencing upregulates protein levels of γ-secretase subunits NCT, PSEN1, PSEN2 and PEN2 (Figure 2, A and B). Similarly, aortic lysates from wild type and Eln(−/−) pups at P0.5 have higher levels of the γ-secretase complex (Figure 2, C and D). Moreover, transverse cryosections of ascending aortas from wild type and Eln(−/−) pups at P0.5 were stained for α-smooth muscle actin (SMA, marker of SMCs) and for PSEN1 or 2, confirming upregulation of the γ-secretase catalytic subunits (Figure 2, E and F). To assess the potential effect of sex on NOTCH3 pathway induction with elastin depletion, we compared the protein levels of NICD3, HES1, PSEN1 and PSEN2 in male and female Eln(−/−) pups. Sex-dependent differences in levels of these proteins or development of elastin aortopathy was not observed (Supplemental Figures 4 and 5). Overall, the NOTCH3 pathway is induced in Eln(−/−) aortas.

Epigenetic modifications influence gene expression by altering chromatin accessibility and play a central role in regulating SMC behavior during vascular development and disease (33, 40). However, epigenetic changes that occur in SMCs in the context of elastin deficiency has not been reported. DNA methylation is a major form of chromatin remodeling in which DNA methyltransferase (DNMT)-mediated transfer of a methyl group to the 5-C position of cytosine renders chromatin inaccessible and silences gene expression (41). Intriguingly, we find that excessive aortic SMCs in Eln(−/−) aortas display dramatically reduced 5-methylcytosine (5mC) mark, suggesting active chromatin remodeling and gene activation in elastin depleted SMCs (Figure 3, A and B). Our analysis of major DNMTs (DNMT1, 3a and 3b) in haSMCs reveal that elastin silencing results in decrease of DNMT1 transcript levels (Figure 3C). Similarly, Western blot analysis of lysates collected from ELN-silenced haSMCs or Eln(−/−) aortas demonstrate reduced DNMT1 protein levels (Figure 3, D-G). To investigate the role of modulated DNA
methylation on expression of \( \gamma \)-secretase genes in SMCs with reduced elastin, we next assessed the status of 5mC at the promoter regions of \( PSEN1 \) and \( PSEN2 \) genes. haSMCs were treated with Scr or ELN-specific siRNA (siELN) and then subjected to 5mC chromatin immunoprecipitation (5mc ChIP). Methylated DNA was immunoprecipitated with a 5mC monoclonal antibody, and recovered DNA was analyzed by qRT-PCR to assess 5mC enrichment at the promoters of \( PSEN1, \) \( PSEN2 \) and \( THS2B \) (the latter being a positive control with constitutive 5mC mark). DNA methylation (5mC) is \(~50\%\) reduced at \( PSEN1 \) and \( PSEN2 \) promoters in elastin-silenced haSMCs (Figure 3H), consistent with increased gene expression.

**Pharmacological \( \gamma \)-secretase inhibition attenuates aortopathy in elastin mutants**

In the wild type aorta, substantial levels of elastin (expressed as the soluble monomer tropoelastin) are initially detectable at E14, and by \(~\)E15, the full complement of SMC layers as in the adult mouse are present (42, 43). \( Eln \) null and wild type aortas are indistinguishable at E15.5, but thereafter the \( Eln^{(-/-)} \) aorta accumulates excess SMCs (3, 20). To test the hypothesis that \( \gamma \)-secretase and the Notch pathway, and specifically NOTCH3, is critical for aortic hypermuscularization and stenosis in \( Eln^{(-/-)} \) mice, we next utilized pharmacological and genetic inhibition. The \( \gamma \)-secretase inhibitor DAPT (N-[(3,5- Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester) acts as a global Notch inhibitor by blocking cleavage of the membrane bound NOTCH intermediate form and hence, the generation and release of the NICD is attenuated. Pregnant dams were injected with DAPT or vehicle on embryonic day (E) 14.5 and E15.5, and transverse sections of the ascending aorta in wild type and \( Eln^{(-/-)} \) pups at P0.5 were stained for CD31 (EC marker) and SMA (Figure 4A). Additionally, the aortas were stained with HES1 (downstream Notch target) confirming Notch pathway inhibition by DAPT
treatment (Figure 4B). Consistent with prior studies (3, 20), $Eln^{(-/-)}$ newbons untreated or
exposed to vehicle in utero display ascending aorta hypermuscularization and stenosis (Figure 4,
C-E). However, DAPT treatment induces a ~2-fold reduction in medial thickness and wall area
and a ~2-fold increase in lumen area of $Eln$ nulls without altering these parameters in wild type
pups. Furthermore, to assess the effect of DAPT-mediated NOTCH inhibition during elastin
haplo-insufficiency, we analyzed the $Eln^{(+/-)}$ aorta which has ~50% reduction in elastin transcript
levels compared to wild type (15) (Supplemental Figure 6A). In agreement with previous studies
(15, 21), newborn $Eln^{(+/-)}$ aortas have a thicker media due to accumulation of additional lamellar
units and SMC layers (Supplemental Figure 6B) without lumen occlusion and stenosis. Newborn
wild type or $Eln^{(+/-)}$ mice were injected daily with DAPT or vehicle from P2.5-5.5, and aortas
were analyzed at P7.5. DAPT treatment reverses increased medial thickness and wall area in the
early postnatal $Eln^{(+/-)}$ aortas (Supplemental Figure 7).

**Notch3 deletion attenuates elastin aortopathy and excessive SMC proliferation in elastin
mutant aorta**

Next, to determine the specific role of NOTCH3 in elastin aortopathy, we analyzed the
effect of Notch3 deletion on the $Eln$ mutant background. In neonatal $Eln^{(+/-)}$ mice, global deletion
of Notch3 results in rescue of medial thickness and medial wall area similar to wild type levels
(Figure 5). Furthermore, on an elastin null background, global Notch3 deletion attenuates
excessive muscularization and stenosis of the aorta at P0.5 as compared to mice wild type for
Notch3 (Figure 6A). Quantitative analysis revealed that similar to DAPT treatment, compound
Notch3$^{(-/-)}$, $Eln^{(-/-)}$ mutants have a 40±8% reduction in medial thickness, 314±73% increase in
lumen area and 21±5% reduction in medial wall area in comparison to $Eln^{(+/-)}$ mice (Figure 6, B-
D). Taken together, these findings from pharmacological and genetic inhibition studies indicate that the pathway involving $\gamma$-secretase and NOTCH3 plays a key role in the pathogenesis of aortic disease in elastin mutants.

We and others have previously shown that in $Eln^{(-/-)}$ aortas, SMCs are hyperproliferative, contributing to increased arterial wall cellularity and stenosis (3, 15, 20). Given that Notch3 deletion mitigates aortic hypermuscularization and stenosis in $Eln^{(-/-)}$ mice (Figure 6, A-D), we next assessed SMC proliferation in aortas of these mice. Dams pregnant with E18.5 embryos were injected with the thymidine analogue 5-ethynyl-2′-deoxyuridine (EdU). Eight hours later, embryos were harvested, and transverse cryosections of ascending aortas were stained for EdU, SMA, CD31 and nuclei (DAPI) (Figure 6E). In comparison to wild type or Notch3($^{(-/-)}$) mice, $Eln^{(-/-)}$ mice display a ~2-fold increase in proliferative aortic SMCs as marked by EdU, SMA and DAPI, and this increase is abrogated in compound Notch3($^{(-/-)}$), $Eln^{(-/-)}$ mice (Figure 6F). A prior study demonstrated that NOTCH3 promotes haSMC survival and proliferation via ERK pathway activation (38). We find that ELN knockdown in haSMCs induces ERK phosphorylation (Supplemental Figure 8, A and B) and that reduction of NOTCH3 in haSMCs decreases transcript levels of pro-survival gene BIRC5 and proliferation-inducing transcriptional factor E2F1 (Supplemental Figure 8C). Overall, these data suggest that inhibition of NOTCH3 pathway during elastin deficiency helps attenuate the aortic phenotype by preventing excessive SMC proliferation.

In addition to SMC hyperproliferation, deficient circumferential growth has been shown to contribute to elastin aortopathy in a model of partial elastin deficiency wherein human elastin is expressed in $Eln^{(-/-)}$ mice (44). Our analysis revealed a minor reduction (~10%) in the external diameter of $Eln^{(-/-)}$ versus wild type and this reduction was abrogated in compound Notch3($^{(-/-)}$).
Eln\(^{+/−}\) mice (Supplemental Figure 9). Taken together, these data suggest that deletion of Notch3 in the Eln null background rescues the aortic phenotype primarily by inhibiting SMC hyperproliferation but also with a minor contribution of improving deficient circumferential growth.

Elastin is critical for lung development, and Eln\(^{+/−}\) mice display dilated distal air sac structures and emphysema (Supplemental Figure 10, A and C), which is likely the major cause of early postnatal death (45, 46). We next assessed whether pharmacological or genetic inhibition of the NOTCH3 pathway could improve lung structure and prolong survival of Eln\(^{+/−}\) mice. Unfortunately, on the Eln\(^{+/−}\) background, DAPT treatment or Notch3 deletion does not rescue lung phenotype or prolong survival (Supplemental Figure 10).

**Elastin deficiency increases NOTCH3-mediated integrin β3 levels**

Our previous studies demonstrated that integrin β3 expression, activation, and signaling are upregulated in the aortic media of Eln\(^{+/−}\) mice and SVAS and WBS patients (3). In elastin mutants, enhanced integrin β3-mediated signaling results in SMC misalignment and hyperproliferation, and pharmacological or genetic inhibition of integrin β3 attenuates aortic hypermuscularization and stenosis (3). Although little is known about the regulation of ITGB3 transcription, NOTCH3 silencing has previously been shown to reduce integrin β3 levels in cultured cells (47). Herein, we initially confirmed these findings by demonstrating that siRNA-mediated knockdown of NOTCH3 reduces levels of ITGB3 mRNA by 53±7% and protein by 64±6% in haSMCs (Figure 7, A-C). To extend these findings to the in vivo setting, the aortas of wild type and Notch3 and/or Eln knockout pups at P0.5 were studied. Similar to results with cultured SMCs, qRT-PCR of isolated aortic RNA revealed a ~50% reduction in Itgb3 transcript
levels in Notch3<sup>−/−</sup> compared to wild type newborns (Figure 7D). In addition, transverse sections of the ascending aorta were stained for integrin β3 (Figure 7E), and aortic lysates were assessed for integrin β3 protein levels by Western blot analysis (Figure 7, F and G). Consistent with our previous investigations (3), integrin β3 staining is upregulated in SMCs of Eln<sup>−/−</sup> aorta compared to that of wild type. More importantly, our results herein indicate that on the Eln<sup>−/−</sup> background, deletion of Notch3 markedly reduces integrin β3 staining.

To further evaluate NOTCH3-mediated regulation of ITGB3 expression in elastin aortopathy, we next investigated the hypothesis that NICD3 binds the ITGB3 gene and that this interaction is enhanced in elastin mutants. haSMCs were treated with Scr or NOTCH3-specific siRNA and then subjected to chromatin immunoprecipitation (ChIP). Protein-DNA complexes were immunoprecipitated with an antibody directed against NICD3 or an isotype matched control antibody. Recovered DNA was analyzed by qPCR to assess NICD3 enrichment at the promoters of the HES1, HEY1 (positive controls) and ITGB3 genes. Our results indicate that NICD3 binds the ITGB3 proximal promoter region in haSMCs, and this interaction is significantly diminished with NOTCH3 knockdown (Figure 7H). Moreover, in a second set of ChIP experiments, we observed that siELN pre-treatment enhances binding of NICD3 to the HES1, HEY1 and ITGB3 promoter region (Figure 7I). Overall, these results indicate that in SMCs in culture and in mice, the NOTCH3 pathway induces integrin β3 levels and NICD3 binds to the ITGB3 promoter, and elastin depletion augments these effects.

**Elastin reduction enhances JAG1 levels in aortic SMCs and ECs**

As the Notch ligand JAG1 is implicated in early arterial morphogenesis (25-28, 47), we next investigated the role of JAG1 during aortopathy in the context of elastin insufficiency.
Elastin silencing in haSMCs results in increased JAG1 mRNA and protein levels (Figure 8, A-C). Similar to promoter regions of PSEN1 and PSEN2, elastin silencing reduces DNA methylation (5mC) at the JAG1 promoter, correlating with increased gene expression (Figure 8D). Additionally, lysates of aortas isolated from Eln(-/-) pups at P0.5 have upregulated levels of JAG1 protein (Figure 8, E and F). Staining of transverse ascending aortic sections from these pups reveals increased JAG1 levels in the hypermuscularized tunica media (Figure 8G). The relevance of these findings to human elastin aortopathies non-syndromic SVAS and WBS was evaluated by assessing JAG1 levels in iPSC-SMCs derived from patients with these diseases as well as in the WBS aorta. SVAS and WBS iPSC-SMCs have greater than 4-fold higher JAG1 protein levels as compared to those cells derived from control human iPSC-SMCs (Figure 8, H and I). Similarly, immunostaining of WBS aortas reveals JAG1 upregulation (Figure 8, J and K). Activation by JAG1 is critical in propagating NOTCH activation through developing layers of the arterial media (28). To further evaluate JAG1-mediated downstream signaling, haSMCs were seeded on recombinant JAG1-coated culture dishes. Our results demonstrate that JAG1 stimulation induces HES1, HEY1 and JAG1 transcript levels (Supplemental Figure 11).

Interestingly, in addition to SMCs, anti-JAG1 staining is also increased in ECs of aortic sections of Eln(-/-) mice at P0.5 (Figure 8G). We postulated that aortic ECs upregulate JAG1 in response to altered ECM lacking elastin, largely derived from SMCs. In large elastic vessels, such as the aorta and pulmonary artery, elastin is predominately produced by SMCs (16, 48, 49), and indeed, our qPCR analysis of cultured cells indicates 300-fold enrichment of ELN transcript in haSMCs compared to haECs (Supplemental Figure 12A). haECs were cultured on ECM derived from haSMCs pre-treated with Scr or ELN-targeted siRNA (Supplemental Figure 12B), and the data indicate that haECs have increased levels of JAG1 transcript (but not other Notch
ligand transcripts) and protein in response to haSMC-derived elastin-deficient ECM (Supplemental Figure 12, C-E). Collectively, these results indicate that elastin deficiency stimulates vascular cell JAG1 expression in cultured human cells and in vivo in mice and humans.

**Jag1 deletion with Acta2-CreERT2, but not Cdh5-Cre, attenuates hypermuscularization and stenosis in elastin mutants**

Given increased JAG1 in vascular cells, we next evaluated the effect of Jag1 specific deletion in ECs and SMCs on the hypermuscularization and stenosis phenotype of Eln mutant mice. For investigation of EC JAG1, Jag1\(^{\text{flox/flox}}\) pups that were also carrying no Cre or the constitutive Cdh5-Cre and either Eln\(^{+/+}\) or Eln\(^{−/−}\) were analyzed (Figure 9A). At P0.5, newborns were genotyped, and the ascending aortas were sectioned transversely and stained for CD31 and SMA. The increased medial thickness and area and reduced lumen area in Jag1\(^{\text{flox/flox}}\) pups of the Eln\(^{−/−}\) background is not altered by the presence of Cdh5-Cre (Supplemental Figure 13, A-C) despite very high [97±2\%] deletion efficiency of Jag1 in ECs as assessed by qRT-PCR (Supplemental Figure 13D), suggesting that EC JAG1 is not requisite for elastin aortopathy. The aorta of Cdh5-Cre, Jag1\(^{\text{flox/flox}}\) adults has previously been shown to have some acellular gaps in the subendothelial SMC layer (47). Consistent with this prior study, our results demonstrate rare gaps in the inner layer of the tunica media of Cdh5-Cre, Jag1\(^{\text{flox/flox}}\) aorta at P0.5; however, at this same time point, the aorta of Eln\(^{−/−}\), Cdh5-Cre, Jag1\(^{\text{flox/flox}}\) mice have markedly more acellular gaps, which are located throughout the media (Supplemental Figure 14).

To determine the role of JAG1 in SMCs during elastin aortopathy, pups with conditional SMC-specific Jag1 deletion on an Eln wild type or null background were generated (Figure 9B).
We injected pregnant dams at E10.5 with 1 mg of tamoxifen and concomitant 0.25 mg of progesterone to minimize the incidence of dystocia (3) and yielding *Jag1* deletion efficiency of 68±14% in aortic SMCs of newborn pups (Supplemental Figure 15). At P0.5, transverse ascending aortic sections were stained for CD31 and SMA (Figure 9B). In comparison to controls, the medial thickness and wall area of *Jag1*(floxflo) *Eln*(−/−) newborns was increased, and the lumen area was decreased; these changes were prevented in *Acta2-CreER*T2, *Jag1*(floxflo), *Eln*(−/−) pups (Figure 9, C-E). Thus, SMC deletion of *Jag1* attenuates hypermuscularization and stenosis in elastin mutants. Taken in their entirety, our findings identify the Notch pathway and specifically, JAG1, NOTCH3 and γ-secretase as key molecular players in the pathogenesis of elastin aortopathy and also as promising therapeutic targets for the human diseases SVAS and WBS (Figure 9F).
DISCUSSION

Obstructive arterial diseases, including atherosclerosis, restenosis, pulmonary hypertension and the genetic elastin arteriopathy SVAS, are characterized by elastic fiber deficiency, degradation and/or fragmentation as well as excess SMCs. The cellular and molecular mechanisms linking elastin defects and hypermuscularization remain incompletely understood which is a major obstacle to the development of novel effective therapies. Indeed, major vascular surgery is the only therapy for the arterial obstruction of SVAS and carries a sizable morbidity and mortality risk. There is a dire need for intense investigation into mechanisms underlying the pathogenesis of elastin aortopathy as insights from these studies have far-reaching impact on diverse vasculoproliferative diseases.

Although no prior investigations have evaluated the function of the Notch signaling pathway during elastin insufficiency, Notch plays a myriad of critical roles during vascular development. Indeed, mice bearing deletion of diverse Notch pathway components have severe cardiovascular defects, many of which result in embryonic lethality (22, 24, 50). Directly relevant to the current study, the Notch pathway regulates arterial SMC differentiation (25, 26, 28, 31). Furthermore, mutations in Notch pathway components in humans cause cardiovascular disorders, such as CADASIL, bicuspid aortic valve and Alagille syndrome (30, 51-53). On the other hand, inhibition of the Notch pathway has been implicated as a potential therapeutic strategy for select vascular diseases, including pulmonary hypertension, tumor angiogenesis and pathological vascular permeability in diabetic retinopathy (29, 50, 54).

In the current study, we utilize a wide array of elastin deficiency models – knockdown in cultured human vascular cells, genetic and pharmacological inhibition in mouse models, and iPSC-SMCs and aortic samples from non-syndromic SVAS and/or WBS patients – to
demonstrate that the JAG1/NOTCH3/γ-secretase pathway is overactive in SMCs with elastin depletion (Figure 1). The catalytic component of the enzyme γ-secretase, PSEN1 or 2, cleaves type 1 transmembrane proteins, such as amyloid precursor protein and NOTCH receptors (55), and interestingly, our data demonstrate that elastin deficiency increases SMC levels of the γ-secretase complex, including PSEN1 and 2 (Figure 2). These results reveal that perturbation of γ-secretase in SMCs plays an important role in elastinopathy.

Epigenetic modifications influence gene expression by altering chromatin accessibility and play a central role in regulating SMC behavior during physiological and pathological conditions including several cardiovascular diseases (33). However, epigenetic regulation in the context of elastin deficiency is previously unexplored, and our data implicate elastin deficiency in modulating the epigenetic landscape of SMCs (Figure 3). Specifically, loss of elastin reduces DNMT1, a pivotal epigenetic regulatory enzyme that catalyzes DNA methylation to induce gene silencing (41). Upon loss of elastin, levels of global as well as locus-specific 5mC mark at promoters of PSEN1, 2 and JAG1 are decreased. These data shed light on molecular mechanisms underlying JAG1/NOTCH3 pathway activation in elastin deficiency. The global hypomethylation of elastin-deficient SMCs is intriguing. One possible explanation is that extensive ECM remodeling associated with elastin deficiency might directly or indirectly regulate DNMT1 levels and trigger genome wide hypomethylation. In addition to expression levels, the enzymatic activity of DNMTs may be altered in Eln(−/−) SMCs. Future investigations into how elastin deficiency regulates DNMT1 and the SMC epigenome promises to reveal further novel insights into the biology of elastin and SMCs.

Pharmacological inhibition of the γ-secretase complex in Eln(−/−) mice reduces the aortic hypermuscular and stenosis phenotype (Figure 4). As described previously (15, 21), the aortas of
Eln\(^{+/−}\) mice display thinner and additional elastic lamellar units with excess SMC layers (Supplemental Figures 6 and 7). Importantly, from a clinical standpoint, our results demonstrate that postnatal treatment with a \(\gamma\)-secretase inhibitor substantially reverses hypermuscularization in Eln\(^{+/−}\) mice (Supplemental Figure 7). In addition, genetic deletion of Notch3 significantly reduces aortic stenosis in elastin nulls and hypermuscularization in both Eln\(^{+/−}\) and Eln\(^{−/−}\) mice (Figure 5 and 6).

Elastin is a critical component of diverse organ systems and is indispensable for lung development. Eln\(^{−/−}\) mice have dilated distal air sacs in the lung and die in the immediate postnatal period (20, 45, 46). NOTCH3 inhibition in Eln\(^{−/−}\) mice neither attenuates lung developmental defects nor prolongs survival (Supplemental Figure 10), indicating that, not surprisingly, inhibiting the NOTCH3 pathway is insufficient to entirely overcome the massive burden of total elastin loss. In contrast to Eln nulls, Eln\(^{+/−}\) mice display normal lung development (56). Thus, targeting the NOTCH3 pathway may be a promising therapeutic strategy for human aortic elastinopathies (i.e., non-syndromic SVAS and WBS) which are caused by ELN haploinsufficiency and generally lack lung parenchymal disease. Because pathological complications of SVAS manifest from infancy onward and often worsen with time (sometimes resulting in sudden death), therapeutic intervention should be considered soon after diagnosis (57, 58).

Elastin is a potent regulator of SMC behavior. Tropoelastin inhibits cultured Eln\(^{−/−}\) aortic SMC proliferation and migration, and an elastin matrix sheath coating of metal stents reduces balloon overexpansion-induced neointimal SMC accumulation and arterial obstruction in porcine coronary arteries (7). Conversely, Eln\(^{−/−}\) aortas accumulate excess SMCs in the subendothelial region of the vessel wall (3, 20), but mechanisms underlying this hypermuscularization are
incompletely understood. Herein, our findings suggest that the NOTCH3 pathway regulates SMC proliferation in *Eln*<sup>−/−</sup> aorta as compound *Notch3<sup>−/−</sup>, Eln<sup>−/−</sup>* mutants display reduced aortic SMC proliferation relative to *Eln*<sup>−/−</sup> and comparable proliferation to wild type mice (Figure 6). It has been previously shown that NOTCH2 and NOTCH3 have opposing effects on SMCs: NOTCH2 inhibits proliferation by reducing MAP kinase activity whereas NOTCH3 promotes cell survival and proliferation via MAP kinase induction (38). In addition, NOTCH3 induces expression of pro-survival genes and prevents apoptosis, while NOTCH2 does not alter apoptosis (38). In agreement with these studies, herein we find that loss of elastin in SMCs upregulates MAP kinase activity (Supplemental Figure 8, A and B) and does not alter NICD2 levels (Supplemental Figure 2) but enhances NICD3 (Figure 1). Consistent with prior studies (38), our data indicate that expression levels of the pro-survival gene *BIRC5* and proliferative gene *E2F1* are reduced by NOTCH3 silencing (Supplemental Figure 8C). Thus, the cumulative effect of elastin deficiency on levels of NOTCH3 (increased) and NOTCH2 (unchanged) may be critical in promoting excessive SMC proliferation and potentially in altering cell survival. Additionally, previous investigations of partial elastin deficiency in which *Eln*<sup>−/−</sup> mice express human elastin showed that impaired outward growth contributes to aortic stenosis (44). Our data suggest that reduced outward growth has a small contribution to the development of stenosis in *Eln*<sup>−/−</sup> model and *Notch3* deletion in elastin null background improves the circumferential growth (Supplemental Figure 9).

We previously reported that integrin β3 expression, activation, and signaling are upregulated in SMCs of elastin-deficient mice and inhibition of integrin β3 attenuates aortic hypermuscularization and stenosis in these mice (3). Although integrin β3 is widely studied, relatively little is known about regulation of Itgb3 transcription. Herein, our initial studies in this
area confirmed prior work (47) that knockdown of NOTCH3 in haSMCs attenuates integrin β3 transcript and protein levels, and we extended this work by showing similar effects in the aorta of Notch3 null mice (Figure 7). Furthermore, our data indicate that in Eln null mice, Notch3 deletion attenuates integrin β3 expression. Most interestingly, ChIP studies demonstrate that NICD3 binds the Itgb3 promoter in haSMCs, and elastin deficiency enhances this interaction.

In addition to the role of the NOTCH3 in elastin aortopathy, we focused on the ligand JAG1, which has been implicated as an important player in arterial wall morphogenesis (25-28, 47). Our results indicate increased expression of JAG1 in aortic SMCs with elastin deficiency in cell culture, in mice and in WBS patients as well as in SVAS and WBS iPSC-SMCs (Figure 8). Mechanistically, elastin deficiency mediates hypomethylation of the JAG1 promoter, resulting in gene activation (Figure 8D). Culturing SMCs on JAG1 coated-dishes results in robust induction of JAG1 and downstream effectors HES1 and HEY1 (Supplemental Figure 11). These results suggest that exposure to JAG1 can mediate rapid activation of the JAG1/Notch pathway positive feed-forward loop in haSMCs (27). Immunohistochemical analysis also revealed enhanced expression of JAG1 in the EC layer of the aorta in Eln(−/−) as compared to wild type mice. The initial study describing Eln(−/−) mice reported a lack of evidence for altered ECs (20). More recently, Wagenseil and colleagues demonstrated that the hypermuscular ascending aorta of Tagln-Cre, Eln(flox/flox) mice lacks intact elastic fibers in the media and has a disrupted internal elastic lamellae (that separates ECs from SMCs) whereas EC-specific deletion of Eln does not alter aortic histology (16). Consistent with these results, we find that ELN transcript levels are highly enriched (>300 fold) in haSMCs as compared to haECs in culture, and interestingly, ECM produced by elastin-silenced SMCs is sufficient to induce EC JAG1 expression (Supplemental
Collectively, our data indicate that JAG1 levels are induced in both ECs and SMCs during elastin deficiency.

Prior studies have demonstrated a key role of EC JAG1 in aortic SMC development and/or maintenance (26, 47). Cdh5-Cre, Jag1(flox/flox) mice are viable with sporadic acellular gaps in the inner layer of the tunica media (47). Our results indicate that Eln(+/−), Cdh5-Cre, Jag1(flox/flox) pups at P0.5 have numerous acellular gaps throughout the aortic tunica media (Supplemental Figure 14); however, this EC loss does not attenuate the stenotic phenotype (Figure 9 and Supplemental Figure 13). Finally, in contrast to Cdh5-Cre-induced EC deletion, SMC-specific deletion of Jag1 with Acta2-CreERT2 attenuates aortic hypermuscularization and stenosis in Eln(+/−) neonates (Figure 9). It has previously been shown that Jag1 deletion with the constitutive Tagln-Cre results in patent ductus arteriosus, defective descending aorta SMC differentiation and early postnatal lethality but does not alter ascending aorta morphogenesis (25). Similarly, we do not observe phenotypic changes in the ascending aorta with SMC-specific deletion of Jag1 (utilizing Acta2-CreERT2) in elastin wild type mice; however, in the Eln(+/−) background, there is a significant reduction in aortic hypermuscularization. These results highlight the critical role of JAG1 in SMC expansion during elastin aortopathy.

Taken together, elastin deficiency in SMCs results in reduction of DNMT1 levels and DNA methylation which induces expression of key Notch pathway genes (JAG1, PSEN1 and PSEN2) and triggers NOTCH3 activation. Elastin-deficient SMCs display increased levels of (i) the Notch ligand JAG1, (ii) γ-secretase complex including PSEN1 and 2, (iii) NICD3 (iv) Notch pathway downstream effectors, including ITGB3 and the HES/HEY family of transcription factors and (v) ERK activation which culminate in excess SMC proliferation and accumulation and stenosis of the ascending aorta (Figure 9F). Additionally, NOTCH3 regulates pro-survival
gene BIRC5, and proliferative factor E2F1 which may provide survival advantage to elastin-deficient SMCs. Our findings reveal critical mechanistic insights into arterial hypermuscularization during elastin insufficiency and identify the JAG1/NOTCH3/γ-secretase pathway as a key mediator of elastin aortopathy. These studies suggest that inhibiting specific components of this pathway in SMCs is a promising therapeutic strategy for human diseases SVAS and WBS and potentially other obstructive arterial diseases associated with elastin insufficiency and excess SMCs.
METHODS

Animal studies and treatments

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Yale University and in accord with NIH Guide for the Care and Use of Laboratory Animals. C57BL/6 wild type [WT]; B6;129S1-Notch3tm1Grid/J [Notch3(+/−) (59)]; and Jag1tm2Grid [Jag1(flox/flox), (60)] mice were from Jackson Laboratory. Elntm1Dyl [Eln(+/−) (20)]; Tg(Cdh5-cre)7Mlia [Cdh5-Cre, (61)]; and Tg(Acta2-cre/ERT2)51Pcn [Acta2-CreERT2 (62)] mice have been previously described. All mice were maintained on C57BL/6 background. Mice were bred and embryos or pups were harvested at different ages with E0.5 considered the time of vaginal plug. All agents were injected intraperitoneally. EdU (Invitrogen, 10 mg/kg body weight) was administered to pregnant dams on E18.5 and embryos were collected 8 hours later. Tamoxifen (Sigma, 1 mg) with concomitant progesterone (Sigma, 0.25 mg) was administered on E10.5, and pups were analyzed immediately after birth at P0.5. Pregnant dams were injected with 1 mg DAPT (Calbiochem) or vehicle (4% DMSO in corn oil) at E14.5 and E15.5, and pups were collected at P0.5. For postnatal analysis, WT or Eln(+/−) mice were injected daily with DAPT (1.5mg/kg body weight) from P2.5-5.5, and pups were collected at P7.5.

Immunohistochemistry

After euthanasia, embryos or pups were fixed in 4% paraformaldehyde for 2 hours, transferred to 30% sucrose in PBS solution, embedded in OCT compound (Tissue Tek), frozen and stored at -80°C. Transverse serial cryosections of ascending aortas of 10 µm thickness were cut starting immediately caudal to the aortic arch and continuing for 200 µm inferiorly and sections near to the aortic arch were utilized for analyses (3-4 sections per mouse). Cryosections
were incubated with blocking solution (5% goat serum, 0.1% Triton X-100 in PBS) and then with primary antibodies diluted in blocking solution overnight at 4°C. On the next day, sections were washed with 0.5% Tween 20 in PBS (PBS-T) and incubated with secondary antibodies diluted in blocking solution for 1 hour. Primary antibodies used were rat anti-CD31 (BD Pharmigen, 553370, 1:100), rabbit anti-JAG1 (Abcam, ab7771, 1:100), rabbit anti-PSEN1 (Cell Signaling, 5643 1:100), rabbit anti-PSEN2 (Cell Signaling, 9979, 1:100), rabbit anti-integrin β3 (Abcam, ab210515, 1:100), HES1 (Cell Signaling, 11988, 1:1000), directly conjugated FITC or Cy3 anti-SMA (Sigma, C6198, 1:300). Secondary antibodies were conjugated to either Alexa 488, 555, 647 (Molecular Probes) or Dylight 555, 649 (Jackson ImmunoResearch) fluorophores and used at 1:500 dilution. DAPI (Sigma, D9542, 1:500) was used for nuclear staining. CF633 hydrazide was used for elastin staining (Sigma SCJ4600037, 1:1000). Click-iT EdU Alexa Fluor 594 Imaging kit (Invitrogen) was used to assess proliferation. For detecting DNA methylation, cryosections were incubated in 2N HCl for 45 minutes at 37°C and washed with 0.1M boric acid prior to incubation in blocking solution for 1 hour. Sections were then stained for 5-Methylcytosine (5mC, Cell Signaling, 28692, 1:100) overnight at 4°C followed by secondary antibody staining as described above. The percentage of medial SMCs with the 5mC mark were scored.

Aortas from patients with WBS (n=5) (3, 9, 63, 64) and human controls (n=11) (as described in Supplemental Table 1) were fixed in formalin, paraffin embedded, and sectioned. Paraffin was removed from sections of human aortas with Histo-Clear (National Diagnostics), and after ethanol washes, sections were rehydrated into water. Rehydrated sections were incubated in boiling antigen retrieval buffer (Dako) for 20 minutes. Sections were allowed to cool at room temperature for 1 hour and then rinsed twice in PBS-T and blocked for 1 hour in
5% goat serum, 0.5% Triton X-100 in PBS. Slides were incubated overnight with anti-JAG1 antibody conjugated to AlexaFluor 647 (Santa Cruz, SC390177, 1:50) at 4°C. The next day, sections were washed with PBS-T and incubated for 1 hour with FITC directly conjugated anti-SMA antibody (Sigma, F3777, 1:500). Propidium iodide (Sigma, P4170, 1:500) was used for nuclear staining.

Quantification of staining intensity and parameters of aortic morphology

Quantifications used Image J software. Fluorescent intensity of immunostaining for JAG1 and SMA on formalin-fixed, paraffin-embedded human aortic sections (10 μm thick) was measured from 5 WBS patients and 11 controls (6-8 fields per sample). Measurements from aortas of WBS patients were normalized to that of age-matched controls as detailed in Supplemental Table 1. The number of SMC nuclei per high power field does not appear to differ substantively between WBS and control groups stratified by age (Supplemental Figure 16). The medial wall thickness from transverse sections of murine aorta were calculated by measuring the distance between the inner aspect of the inner and the outer aspect of the outer SMA+ medial layers (8 measurements per section and 3-4 sections per mouse). Medial and lumen areas were calculated by measuring the area of SMA staining and the area interior to CD31 staining, respectively (3-4 sections per mouse). The external diameter was measured on aortic transverse sections as the length of a line having endpoints at the outer aspects of the outermost SMA+ medial layer and passing through the center of the lumen (measurements of 2 perpendicular lines per section and 3-4 sections per mouse). All morphometric analyses of the aorta were done in a blinded fashion. One potential limitation is that because perfusion fixation was not performed in vivo, lumen loss measured on sections could be an artifact of postmortem vessel constriction.
Patient-derived iPSC-SMC generation

Human undifferentiated iPSCs reprogrammed from skin fibroblasts of a WBS patient [WS1-iPSC line C from (65, 66)], an ELN mutant non-syndromic SVAS patient [ELN1 from (66)] and a control human [CT2 from (66)] were used to generate iPSC-SMC progenitor cell lines via an embryoid body stage (65, 66). The age and sex of iPSC donors are provided in Supplemental Table 2. As described previously (67), the SMC progenitor cells were expanded in Matrigel-coated 6-well plates in smooth muscle growth medium (Medium 231, growth supplement) (Life Technologies). Cells were passaged when they reached 80-90% confluence. To induce differentiation, the iPSC-SMC progenitors were trypsinized and plated in smooth muscle differentiation medium (Medium 231, differentiation supplement) (Life Technologies) on 0.1% gelatin-coated 6-well plates for 6 days. RNA was isolated before and after SMC differentiation and utilized for qRT-PCR analysis of SMC markers. Protein lysates from the differentiated iPSC-SMCs were collected and utilized for Western blotting.

Cell culture and siRNA-mediated knockdown

haSMCs (Lonza) or haECs (ScienCell) were cultured up to passage 6 in M199 medium supplemented with 10% FBS, EGF and FGF (PromoCell) or complete EC medium (ScienCell), respectively. For gene silencing, siRNA was transfected as described previously (68). Briefly, haSMCs were transfected with Lipofectamine 2000 (Life Technologies) containing siRNA targeted against ELN (Dharmacon, 50 nM) or NOTCH3 (Origene, 50 nM) or Scr RNA for 6 hours. Cells were then washed in M199 and cultured for 72 hours prior to collection for qRT-PCR or Western blot analysis.
**Quantitative real-time RT-PCR**

For cultured haSMCs or haECs, RNA was isolated with the RNeasy Plus Kit (Life Technologies). For newborn mice, PBS was perfused through the left ventricle, the entire aorta from the root to the iliac arteries was dissected and aortic RNA was extracted with mechanical homogenization in Trizol (Invitrogen) and PureLink RNA columns (Invitrogen). The isolated RNA was reverse transcribed with the iScript cDNA Synthesis Kit (BioRad), and qRT-PCR was performed on a CFX96 Real Time System (BioRad) using SsoFast EvaGreen supermix (BioRad) and primer pairs as per Supplemental Table 3. Normalized mRNA levels are relative to 18S rRNA for cultured cells and to 18S rRNA or Gapdh for murine aortas.

**Western blot**

Lysates of cultured cells were prepared by solubilizing cells in boiling 1.5X Laemmli sample buffer at 95°C for 10 minutes. Lysates from aortas were prepared by pooling aortas from two pups for each genotype per sample and mechanically lysing in 1.5X Laemmli sample buffer on ice with a glass pestle tissue homogenizer (Pyrex). Aortic lysates were then centrifuged at 16,000g for 2 minutes at 4°C, supernatants were collected, and protein concentration was determined by BCA assay (Thermo Scientific). Protein samples from cultured cells or aortas were resolved by 7–15% SDS-PAGE, transferred to Immobilon PVDF membranes (Millipore), blocked with 5% nonfat dry milk or bovine serum albumin, washed in Tris-buffered saline, 0.1% Tween 20 (TBS-T) and probed with primary antibodies overnight at 4°C. Membranes were incubated with HRP-conjugated secondary antibodies (Dako), washed in TBS-T, developed with Supersignal West Pico Maximum Sensitivity Substrate (Pierce) and analyzed with the G:BOX
imaging system (Syngene). Primary antibodies used for Western blot analysis were rabbit anti-NOTCH3 (Cell Signaling, 2889, 1:500), rabbit anti-NOTCH2 (Cell Signaling, 5732, 1:500), rabbit anti-DNMT1 (Cell Signaling, 5032, 1:1000), rabbit anti-JAG1 (Cell Signaling, 70109, 1:1000), rabbit anti-HES1 (Cell Signaling, 11988, 1:1000), rabbit anti-NCT (Cell Signaling, 5665, 1:1000), rabbit anti-PSEN1 (Cell Signaling, 5643, 1:1000), rabbit anti-PSEN2 (Cell Signaling, 9979, 1:1000), rabbit anti-PEN2 (Cell Signaling, 8598, 1:1000), rabbit anti-integrin β3 (Abcam, 197662, 1:1000), rabbit anti-pERK (Cell Signaling, 9101, 1:1000), rabbit anti-ERK (Cell Signaling, 9102, 1:1000), rabbit anti-GAPDH (Cell Signaling, 2218, 1:2500), rabbit anti-human ELN (generated against human aortic alpha elastin; 1:500) and rabbit anti-mouse ELN (raised against exons 6-17 of recombinant mouse tropoelastin; 1:500). All anti-ELN antibodies were provided by Dr. Robert Mecham (14, 69).

5-Methylcytosine chromatin immunoprecipitation (5mC ChIP)

Methylated DNA Immunoprecipitation (MeDIP) was carried out using the protocol published earlier (70). Briefly, enrichment of methylated DNA was performed using the MeDIP kit (C02010021, Diagenode) as per the manufacturer’s instructions. A total amount of 1 µg of genomic DNA was isolated from haSMCs pretreated with Scr or siELN and sheared to 200-500 bp using the Covaris sonicator. Methylated DNA was captured by incubation with anti-5mC monoclonal antibody coupled to magnetic beads overnight at 4°C. The beads were washed and methylated DNA was eluted. The recovered DNA and input fractions were analyzed by qRT-PCR to assess enrichment of the methylated DNA at PSEN1, PSEN2 and JAG1 gene promoters. The following forward and reverse primer pairs spanning canonical CSL binding motifs upstream of the transcription start site were used: PSEN1 5’-GTTCTCCCCGCAATCGTTTTC-3’
and 5’-CACCGTTGTGTCATTTCCG; PSEN2 5’-CCCCAGTGAGGAGGGAAC-3’ and 5’-CTCCAGCGGAGTTACGCA-3’; JAG1 5’-GTAGAAGAACCAGGCCCCA-3’ and 5’-AGCAACGACCCCTTTCAAGT-3’. The location of the PCR amplification product with PSEN1 primers is chr14:73,136,336-73,136,451, with PSEN2 primers is chr1:226,870,512-226,870,633 and with JAG1 primers is chr20:10,673,792-10,674,673. All samples were performed in at least triplicate, from four independent experiments and data were calculated by % input method. The primer pairs for THS2B promoter (positive control with constitutive 5mC mark) were included in the MeDIP kit.

Imaging

Fluorescent images of aortic sections were acquired with a confocal microscope (PerkinElmer UltraView Vox Spinning Disc). Brightfield images of H&E staining were captured using inverted microscopes (Eclipse 80i and Eclipse TS100, Nikon). Volocity software (PerkinElmer) and Adobe Photoshop were used to process images.

Statistics

Student’s t-test and multifactor ANOVA with Tukey’s post hoc test were used to analyze the data after testing for normality and equal variance using Graph Pad Prism (version 6.03). Statistical significance threshold was set at $p \leq 0.05$. All data are presented as mean ± standard deviation (SD).

Study approvals
All procedures involving human aorta tissue isolated at the time of surgery or autopsy were approved by the Institutional Review Boards of Yale University (#2000020632), Stanford University (#12726) and University of Pittsburgh (PRO10020125) and the New England Organ Bank and complied with all relevant ethical regulations. For patient iPSC generation, all procedures were approved by the Institutional Review Board of the Hospital for Sick Children in Toronto, Canada (#100001232) and the Stem Cell Oversight Committee of the Canadian Institutes of Health Research. All participants, parents or legal guardians provided written informed consent to participate in the study. All mouse experiments were approved by the Institutional Animal Care and Use Committee at Yale University.
AUTHOR CONTRIBUTIONS

J.M.D., R.C., A.N., F.Z.S., Z.F., A.M., K.M. and D.M.G. conceived of and designed experiments and J.M.D., R.C., A.N., J.S. and F.Z.S. performed them. C.K., J.E and S.M. provided SMC progenitors derived from human iPSCs; R.K.R., Z.U. and G.T. provided human aortic samples and R.M. provided anti-elastin antibody. J.M.D. and D.M.G. analyzed the results, prepared the figures and wrote the manuscript. All authors reviewed and provided input on manuscript. Competing interests: none. Data and materials availability: Upon reasonable request, iPSC-derived SMC progenitor lines are available from J.E. and S.M. and anti-elastin antibodies are available from R.M. All other materials reported in this manuscript are commercially available. All the data are available in the main text and supplementary information.

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REFERENCES


Figure 1. Upregulation of NOTCH3 pathway in human and mouse elastin mutants.

(A) Schematic of NOTCH3 pathway. Upon binding ligand (JAG or DLL) expressed by a neighboring cell, the transmembrane NOTCH full length receptor is cleaved by tumor necrosis factor-α-converting enzyme (TACE), producing an intermediate form that remains membrane bound but lacks the extracellular region. This intermediate form is further cleaved by γ-secretase to release the Notch intracellular domain ( NICD ) which translocates to the nucleus, forms a complex with transcription factor CSL and co-activator Mastermind-like (MAML) and induces expression of target genes (e.g., HEY and HES family members).

(B-D) Human aortic SMCs were treated with scrambled (Scr) or ELN-specific siRNA (siELN), and lysates were analyzed. In (B), histogram depicts levels of indicated transcripts relative to 18S rRNA in lysates as assessed by qRT-PCR and normalized to Scr treatment (n=3). Western blots probed for ELN, NOTCH3 (full length and intermediate forms), NICD3, HES1 and GAPDH are shown in (C) with densitometry of protein bands relative to GAPDH and normalized to Scr in (D; n=3-5).

Student’s t-test, *p<0.05, **p<0.01, ****p<0.0001 vs. Scr.

(E, F) Aortic lysates from wild type (WT) or Eln<sup>−/−</sup> mice at postnatal day (P) 0.5 (two aortas pooled per genotype for each n) were subjected to Western blots for ELN, NICD3, HES1 and GAPDH (E) with densitometry of protein bands relative to GAPDH and normalized to WT (F). n=3-6 mice. Student’s t-test, *p<0.05, **p<0.01, ***p<0.001 vs. WT.

(G, H) iPSC-derived SMC progenitors from WBS or non-syndromic SVAS patients or controls were differentiated into SMCs. Protein levels of ELN, NICD3, HES1 and GAPDH in these iPSC-SMCs were assessed by Western blot in (G) with densitometric analysis of ELN, HES1 and NICD3 normalized to GAPDH (H; n=3). One-way ANOVA with Tukey’s post hoc test, *p<0.05, **p<0.01, ***p<0.001. All data are averages ± SD. Gels and blots for HES1 and GAPDH were run contemporaneously (C, E and G).
Figure 2

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Figure 2. Enhanced aortic $\gamma$-secretase levels with elastin deficiency. (A, C) Lysates were subjected to Western blot for $\gamma$-secretase subunits NCT, PSEN1, PSEN2 and PEN2 and for GAPDH. In (A), human aortic SMCs were pre-treated with Scr or siELN RNA, and in (C) aortas of two wild type (WT) or $Eln^{-/-}$ mice at P0.5 were pooled. (B, D) Densitometry of protein bands in (A) and (C) relative to GAPDH and normalized to Scr in (B) or WT in (D; n=3). Student’s $t$-test, *$p<0.05$, **$p<0.01$ vs. Scr (B) or WT (D). Data are averages $\pm$ SD. (E, F) Ascending aortic cross-sections of WT and $Eln^{-/-}$ mice at P0.5 stained for $\alpha$-smooth muscle actin (SMA, marker of SMCs), nuclei (DAPI) and either PSEN1 in (E) or PSEN2 in (F). n=3 mice. Lu, lumen. Scale bars, 25 $\mu$m.
Figure 3

A

B

C

D

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H
Figure 3. Elastin deficiency reduces DNA methylation (5mC) epigenetic mark and downregulates DNMT1 levels. (A) Transverse sections of ascending aorta from WT or Eln<sup>−/−</sup> pups at P0.5 stained for 5-methylcytosine (5mC), SMA, and propidium iodide (PI, nuclei). Lu, lumen. Scale bar, 50 μm. (B) Histogram representing the percentage of 5mC<sup>+</sup> SMCs in (A; n=3 mice). Student’s t-test, **<i>p</i> < 0.01 vs. WT. (C-E) haSMCs were pretreated with Scr or siELN and then lysates were analyzed. In (C), histogram depicts transcript levels of DNMT1, DNMT3A and DNMT3B from qRT-PCR relative to 18S rRNA and normalized to Scr (n=6). Student’s t-test, **<i>p</i> < 0.01 vs. Scr. (D) Western blots for DNMT1 and GAPDH and densitometry of protein bands in (E) relative to GAPDH and normalized to Scr (n=4). Student’s t-test, ***<i>p</i> < 0.001 vs. Scr. (F, G) Aortic lysates from wild type (WT) or Eln<sup>−/−</sup> mice at P0.5 subjected to Western blot for DNMT1 and GAPDH (F) with densitometry of protein bands relative to GAPDH and normalized to WT (G; n=3 mice). Student’s t-test, **<i>p</i> < 0.01 vs. WT. (H) Genomic DNA was isolated from haSMCs pretreated with Scr or siELN and then subjected to methylated DNA chromatin immunoprecipitation (5mC ChIP). Histogram represents 5mC levels at the promoter regions of <i>PSEN1</i>, <i>PSEN2</i> or <i>THS2B</i> (constitutive 5mC positive control) as assessed by qRT-PCR and normalized to Scr (n=4). Student’s t-test, **<i>p</i> < 0.01 , ***<i>p</i> < 0.001 vs. Scr. All data are averages ± SD.
Figure 4

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C

![Image](Media thickness (µm))

D

![Image](Lumen area (10^3 µm^2))

E

![Image](Media area (10^3 µm^2))

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**Figure 4**

- **A**: Comparison of SMA and CD31 expression in WT and Eln(-/-) mice treated with vehicle (Veh) or DAPT.
- **B**: Comparison of HE and CD31 expression in WT and Eln(-/-) mice treated with vehicle (Veh) or DAPT.
- **C**: Graph showing media thickness in WT and Eln(-/-) mice treated with vehicle (Veh) or DAPT.
- **D**: Graph showing lumen area in WT and Eln(-/-) mice treated with vehicle (Veh) or DAPT.
- **E**: Graph showing media area in WT and Eln(-/-) mice treated with vehicle (Veh) or DAPT.
Figure 4. Inhibition of γ-secretase attenuates hypermuscularization and stenosis in Eln<sup>−/−</sup> mice. (A) Pregnant dams were injected on both E14.5 and E15.5 with either vehicle (4% DMSO in corn oil) or γ-secretase inhibitor DAPT (1 mg). Transverse sections of the ascending aorta from pups at P0.5 of indicated genotype and treatment were stained for SMA (SMC marker) and CD31 (EC marker). (B) Transverse sections in (A) stained for HES1, SMA, CD31 and nuclei (DAPI). Lu, lumen. Scale bars, 100 μm (A) and 10 μm (B). (C-E) Histograms represent medial thickness (C), lumen area (D) and medial wall area (E) of ascending aortas from (A). n=4-6 mice. Two-way ANOVA with Tukey’s post hoc test, ***p<0.001, ****p<0.0001. All data are averages ± SD.
Figure 5

A

WT  Notch3(-/-)  Eln(+/-)  Eln(+/-), Notch3(-/-)

B

WT  Notch3(-/-)  Eln(+/-)  Eln(+/-), Notch3(-/-)

C

Media thickness (µm)

D

Lumen area (10^3 µm^2)

E

Media area (10^3 µm^2)
Figure 5. *Notch3* deletion in *Eln*<sup>+/−</sup> mutants reduces aortic muscularization. (A, B)

Transverse sections of the ascending aorta from pups at P0.5 of indicated genotype were stained for SMA and CD31 in (A) and for ELN, SMA and nuclei (DAPI) in (B). Lu, lumen. Scale bars, 100 µm (A) and 10 µm (B). (C-E) Histograms represent medial thickness (C), lumen area (D) and medial area (E) from (A). n=5 mice per group. One-way ANOVA with Tukey’s *post hoc* test, ***p<0.001, ****p<0.0001. All data are averages ± SD.
Figure 6

A

WT          Notch3(-/-)          Eln(-/-)          Eln(-/-), Notch3(-/-)

SMA         CD31

B

WT          Notch3(-/-)          Eln(-/-)          Eln(-/-), Notch3(-/-)

Media thickness (µm)

C

WT          Notch3(-/-)          Eln(-/-)          Eln(-/-), Notch3(-/-)

Lumen area (10^3 µm^2)

D

WT          Notch3(-/-)          Eln(-/-)          Eln(-/-), Notch3(-/-)

Media area (10^3 µm^2)

E

WT          Notch3(-/-)          Eln(-/-)          Eln(-/-), Notch3(-/-)

E18.5       EdU                   SMA

F

WT          Notch3(-/-)          Eln(-/-)          Eln(-/-), Notch3(-/-)

EdU^+ SMA^+ DAPI^+ (%)

Media thickness (µm)

Lumen area (10^3 µm^2)

Media area (10^3 µm^2)

EdU^+ SMA^+ DAPI^+ (%)
Figure 6. On the *Eln*(−/−) background, Notch3 deletion attenuates hypermuscularization and stenosis and reduces SMC proliferation. (A) Transverse sections of the ascending aorta from pups at P0.5 of indicated genotype were stained for SMA (SMC marker) and CD31 (EC marker). Lu, lumen. Scale bar, 100 µm. (B-D) Histograms represent medial thickness (B), lumen area (C) and medial wall area (D) of ascending aortas from (A). n=4-5 mice. One-way ANOVA with Tukey’s post hoc test, **p<0.01, ***p<0.001, ****p<0.0001. (E) EdU was injected in pregnant dams at E18.5, and eight hours later, embryos of indicated genotypes were collected. Cryosections of ascending aortas were stained for EdU, SMA, CD31 and nuclei (DAPI). Proliferative SMCs were marked by EdU+ SMA+ DAPI+ cells. Lu, lumen. Scale bar, 25 µm. (F) Histogram represents the percentage of SMCs that are proliferative in (E). n=3 mice. One-way ANOVA with Tukey’s post hoc test, **p<0.01. All data are averages ± SD.
Figure 7

A. mRNA levels of NOTCH3 and ITGB3.

B. Western blot showing NICD3, β3, and GAPDH.

C. Comparison of mRNA levels between Scr and siNOTCH3.

D. Comparison of mRNA levels between WT and Notch3(-/-).

E. Immunofluorescence images showing expression of Lu, DAPI, and SMA.

F. Western blot showing ITGB3 protein levels.

G. Comparison of ITGB3 protein levels between WT, Eln(-/-), Notch3(-/-), Eln(-/-), Notch3(-/-).

H. % Input of HES1, HEY1, ITGB3 with Ab: NICD3, β3, IgM.

I. % Input of HES1, HEY1, ITGB3 with Ab: NICD3, IgM.
Figure 7. NOTCH3 regulates integrin β3, and elastin silencing promotes NICD3 binding to ITGB3. (A-C) haSMCs were treated with Scr or siNOTCH3 RNA, and then cell lysates were analyzed. In (A), histogram represents transcript levels of NOTCH3 and ITGB3 relative to 18S rRNA as assessed by qRT-PCR and normalized to Scr treatment (n=3). Western blots for NICD3, integrin β3 and GAPDH are shown in (B) with densitometry of protein bands relative to GAPDH and normalized to Scr in (C; n=3). Student’s t-test, **p<0.01, ***p<0.001, ****p<0.0001 vs. Scr. (D) RNA isolated from aortas of wild type (WT) or Notch3(-/-) pups at P0.5 was subjected to qRT-PCR. Histogram represents mRNA levels of Notch3 and Itgb3 relative to 18S rRNA and normalized to WT (n=4 mice). Student’s t-test, *p<0.05, **p<0.01 vs. WT. (E) Ascending aortic transverse sections of indicated genotypes at P0.5 stained for integrin β3, SMA and nuclei (DAPI) (n=3 mice). Lu, lumen. Scale bar, 25 μm. (F, G) Aortic lysates from mice of indicated genotype at P0.5 subjected to Western blot for integrin β3 and GAPDH (F) with densitometry of protein bands relative to GAPDH and normalized to WT (G; n=4-5 mice). One-way ANOVA with Tukey’s post hoc test, *p<0.05, ***p<0.001, ****p<0.0001. (H, I) Chromatin immunoprecipitation was performed with antibodies directed against NICD3 or IgM control in human aortic SMCs pre-treated with Scr or siNOTCH3 in (H) and Scr or siELN in (I). qRT-PCR was then conducted with primers specific for regions upstream of the HES1, HEY1 or ITGB3 transcription start sites. Graph shows qRT-PCR results calculated by % input method (n=3). One-way ANOVA with Tukey’s post hoc test, *p<0.05, **p<0.01, ***p<0.001. All data are averages ± SD.
Figure 8. Elastin deficiency in SMCs induces JAG1 upregulation. (A-C) haSMCs were treated with Scr or siELN RNA, and then lysates were analyzed. In (A), histogram represents ELN and JAG1 transcript levels relative to 18S rRNA as assessed by qRT-PCR and normalized to Scr treatment (n=3). Western blots for JAG1 and GAPDH are shown in (B) with densitometry of protein bands relative to GAPDH and normalized to Scr in (C; n=3). Student’s t-test, *p<0.05, ****p<0.0001 vs. Scr. (D) Methylated DNA (5mC) ChIP from haSMCs pretreated with Scr or siELN. Histogram represents 5mC levels at promoter regions of JAG1 or THS2B (positive control) by qRT-PCR and normalized to Scr (n=4). Student’s t-test, **p<0.01 vs. Scr. (E, F) Aortic lysates from wild type (WT) or Eln(−/−) mice at P0.5 were subjected to Western blot for JAG1 and GAPDH (for each blot, two aortas were pooled per genotype) with densitometry of JAG1 protein bands relative to GAPDH and normalized to WT are shown (n=6 mice). Student’s t-test, **p<0.01 vs. WT. (G) Transverse sections of ascending aorta from WT and Eln(−/−) mice at P0.5 were stained for JAG1, CD31, SMA and nuclei (DAPI). n=3 mice. Lu, lumen. Scale bar, 25 μm. (H, I) Protein levels of JAG1 and GAPDH in iPSC-SMCs derived from control or WBS or SVAS patients as assessed by Western blot with densitometric analysis of JAG1 normalized to GAPDH (n=3). One-way ANOVA with Tukey’s post hoc test, ***p<0.001. (J) Aortic sections from a WBS male patient (46 years old) and control male (53 years old) stained for JAG1, SMA and nuclei (PI). Scale bar, 50 μm. (K) Column scatter plot represents fluorescent intensity of JAG1 and SMA immunostaining in aortic sections of WBS patients (n=5) normalized to age-matched controls (n=11). Intensity was quantified on 8-10 microscopic fields per patient. Student’s t-test, **p<0.01 vs control. All data are averages ± SD.
Figure 9

A

B

C

D

E

F

Downregulated or
Upregulated in Eln mutant aorta
Targets that regulate aortic stenosis
Figure 9. In elastin mutant mice, deletion of Jag1 in SMCs but not ECs, attenuates muscularization and stenosis. (A, B) Jag1(flox/flox) pups also carrying no Cre, Cdh5-Cre or Acta2-CreER^{T2} and either wild type or null for Eln were collected at P0.5. Transverse sections of the ascending aorta indicated genotypes were stained for SMA and CD31. In (B), pregnant dams were injected with tamoxifen at E10.5. Lu, lumen. Scale bars, 100 μm. (C-E) Medial thickness (C), lumen area (D) and medial area (E) of sections of ascending aortas from mice of genotypes as in (B). n=5-6 mice. One-way ANOVA with Tukey’s post hoc test, *p<0.05, **p<0.01, ****p<0.0001. Data are averages ± SD. (F) Schematic of study findings are shown. During elastin deficiency, reduction in DNMT1-mediated DNA methylation results in expression of PSEN1, PSEN2 and JAG1 genes. These gene products culminate in increased NICD3 and activation of NOTCH3 pathway members (downstream genes, including JAG1, ITGB3, HES1 and HEY1) leading to SMC hyperproliferation, hypermuscularization and stenosis. Pharmacological inhibition of γ-secretase complex (via DAPT) or genetic deletion of Notch3 or SMC Jag1 attenuates aortic phenotype of Eln^{−/−} mice. Note, we previously found that pharmacological or genetic inhibition of ITGB3 has similar effects (3).